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Review

Biosynthesis of isoprenoids via the non-mevalonate pathway

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Abstract. The mevalonate pathway for the biosynthesis of the universal terpenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), is known in considerable detail. Only recently, the existence of a second mevalonate-independent pathway for the biosynthesis of IPP and DMAPP was detected in plants and certain eubacteria. Experiments with ^{13}C and/or ^{2}H-labelled precursors were crucial in the elucidation of this novel route. The pathway is essential in plants, many eubacteria and apicomplexan parasites, but not in archaea and animals. The genes, enzymes and intermediates of this pathway were rapidly unravelled over the past few years. Detailed knowledge about the mechanisms of this novel route may benefit the development of novel antibiotics, antimalarials and herbicides.

Key words. Isoprenoid biosynthesis; terpenes; retrobiosynthetic analysis; isopentenyl diphosphate; dimethylallyl diphosphate; mevalonate; deoxyxylulose; methylerythritol.

Introduction

The structural diversity of terpenes is immense, including acyclic, monocyclic and polycyclic compounds [1]. Numerous terpenoids have important medical aspects. Thus, cholesterol is an essential component of vertebrate membranes, but excessive serum cholesterol levels are an important factor in the natural history of cardiovascular disease. Cholesterol serves as biosynthetic precursor of numerous steroid hormones, including glucocorticoids, mineralocorticoids, estrogens, gestagens and androgens, and a wide variety of synthetic analogs are used for therapeutic purposes and as contraceptives. The lipid-soluble vitamins A, D, E and K are also medically important members of the large terpene family.

In recent years, the yew toxin, taxol, has become one of the most important cytostatic agents for the therapy of malignant tumours such as mammary and ovarian carcinoma [2]. More recently, the carotenoids lutein and lycopene have been registered for use as oncopreventive agents [3, 4].
Certain proteins involved in cell cycle regulation are recruited to the plasma membrane by covalent modification with lipophilic side chains of terpenoid origin, and the enzymes involved in that posttranslational modification are potential targets for cancer chemotherapy [5, 6].
In light of the many diverse functions of terpenes, it is not surprising that their biosynthesis has been the subject of intense investigation for more than 4 decades. The classical studies performed in the research groups of Bloch, Cornforth and Lynen showed that all carbon atoms of isopentenylallyl diphosphate (IPP) (**2**) and dimethylallyl diphosphate (DMAPP) (**3**) are derived from acetyl-coenzyme A (CoA) (**1**) in yeast and animal cells (for review, see

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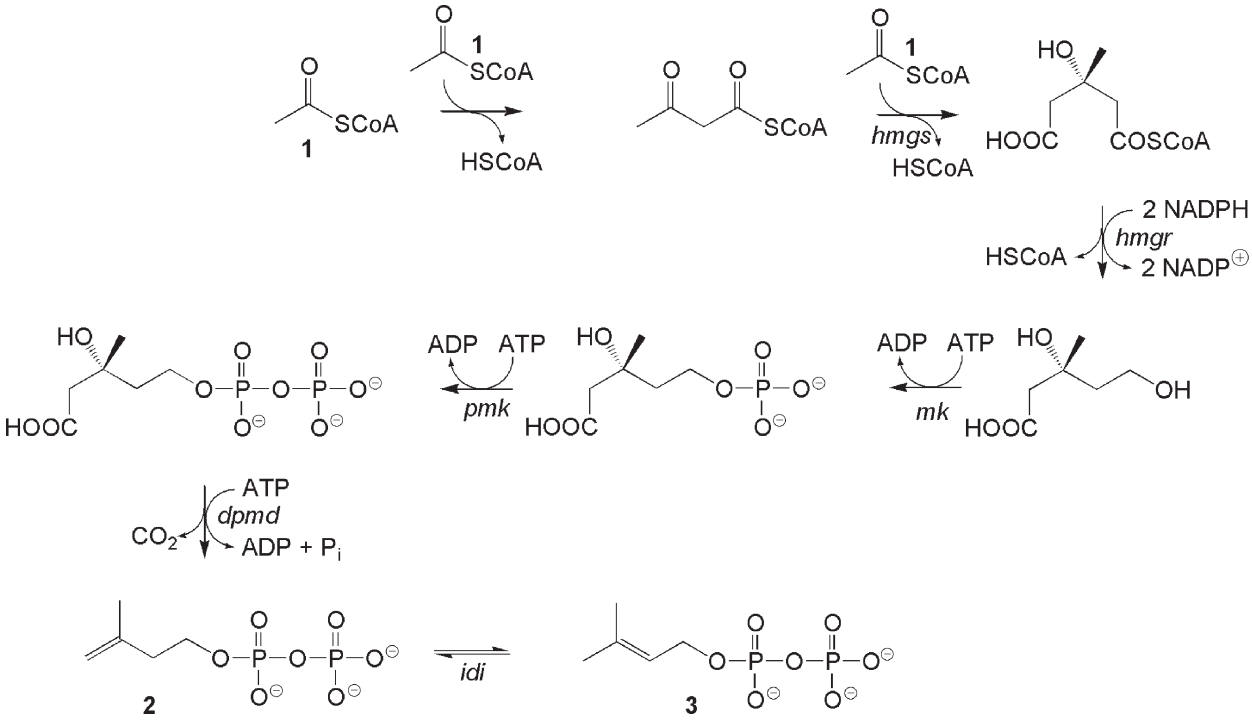

Figure 1. Mevalonate pathway of isopentenyl diphosphate (IPP, **2**) and dimethylallyl diphosphate (DMAPP, **3**) biosynthesis.

[7–10]). (fig. 1). An important spinoff derived from this classical work was the development of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins), which are widely used for the prevention and therapy of cardiovascular disease by lowering cholesterol levels in blood [11, 12].

Discovery of an alternative pathway

For several decades, the mevalonate pathway was believed to be the unique source of isoprenoid building blocks, and the existence of a separate pathway conducive to IPP and DMAPP was sturdily ignored for reasons which will be discussed later. Ultimately, the impossibility to reconcile certain biosynthetic data with the mevalonate paradigm was independently recognized by Rohmer, Arigoni and their respective co-workers [13–15]. Rohmer and his co-workers studied the incorporation of various [$^{13}C_1$]glucose isotopomers and [^{13}C]acetate isotopomers into hopanoids in certain eubacteria and found the location of the label in the target molecules to be strangely at odds with the mevalonate prediction [13]. Arigoni and Schwarz, in seedlings of the tree *Ginkgo biloba* observed the joint transfer of three contiguous carbon atoms from [U-$^{13}C_6$]glucose into ginkgolides, this finding is not compatible with a mevalonate origin of ginkgolides; since mevalonate is assembled from two-carbon precursors [14]. Moreover, with [1-$^{13}C_1$]glucose as a precursor, Arigoni and Broers showed that the labelling pattern of ubiquinone formed in *Escherichia coli* cannot be explained in terms of the mevalonate pathway [15]. The details of these early studies have been reviewed repeatedly [16–19] and will not be repeated here. The unexpected isotope patterns in the experiments listed above were best explained by condensation of 'activated acetaldehyde', and a triose phosphate as an initial reaction step of an alternative biosynthetic pathway [15]. This hypothesis was confirmed subsequently in studies with mutants of *E. coli* [20].

A decisive breakthrough in the elucidation of the unknown pathway was the discovery that isotope-labelled 1-deoxy-D-xylulose is incorporated by *E. coli* cells into the terpenoid side chain of ubiquinone with extraordinary efficacy [15]. Earlier work had already shown that the carbon skeleton of 1-deoxy-D-xylulose can be incorporated into pyridoxal (vitamin B_6) [21] and into the thiazole ring of thiamine (vitamin B_1) [22–24]. Thus, 1-deoxy-D-xylulose or a derivative thereof appeared to qualify as the branching intermediate for the biosynthetic pathway of vitamins B_1 and B_6 and a novel, non-mevalonate pathway of isoprenoid biosynthesis. Later on, it was shown that the 5-phosphate of 1-deoxy-D-xylulose, derived from pyruvate and D-glyceraldehyde 3-phosphate, serves as the branching intermediate (see below).

The biosynthetic origin of different plant terpenoids

Well above 20,000 plant terpenes have been reported. A subgroup comprising sterols, carotenoids, chlorophylls,

quinones and dolichol serves essential functions in all plants, but the majority of plant terpenes can be classified as secondary metabolites, serving at best specialized functions.

As described above, all plant terpenoids studied up to about 1990 had been assigned a mevalonate origin on the basis of isotope incorporation experiments with mevalonate or acetate. Although these experiments typically proceeded with low incorporation levels attributed to permeability barriers, the label distribution, when analysed carefully, was in line with the mevalonate paradigm.

In light of the more recent evidence described below, it is now clear that in a strange way, these earlier results were experimentally correct yet inappropriately interpreted. In the meantime, the coexistence of the mevalonate and the non-mevalonate pathway in plants has been demonstrated (for details see below). The recent studies have established that in plants the compartmental separation between the two isoprenoid pathways is not an absolute one. Minor amounts of one or more unidentified metabolites common to both pathways can be exchanged in both directions via the plastid membranes. Thus, label of 1-deoxyxylulose-derived isoprenoid moieties can be diverted to the cytoplasm, where it can become part of sterol molecules [25]. Likewise, a small fraction of isoprenoid moieties derived from the mevalonate pathway find their way into the plastid compartment where they become part of mono- and diterpenes which are predominantly obtained via the plastid-based deoxyxylulose pathway [14, 26–28].

A powerful strategy for quantitative assessment of the differential contribution of the two isoprenoid pathways for the biosynthesis of individual terpenes uses ^{13}C-labelled glucose as precursor for metabolic studies with whole plants, plant tissues or cultured plant cells [29–43]. Since glucose is a general intermediary metabolite, the isotope from the proffered carbohydrate can be diverted to virtually all metabolic compartments and intermediates in plant cells. On the basis of glycolytic pathways in plants, the transfer of ^{13}C-label from glucose to IPP/DMAPP via mevalonate or 1-deoxy-D-xylulose 5-phosphate can be predicted (fig. 2). It is obvious that the labelling patterns of the terpenoid precursors and their downstream products will be determined by the specific contributions of the two terpenoid pathways. Using ^{13}C nuclear magnetic resonance (NMR) spectroscopy, the ^{13}C enrichment for all non-isochronous carbon atoms can be determined with high precision. Biosynthetic information can then be derived from the positional aspects of the label distribution in the target molecule rather than from the net transfer of isotope. This procedure is in sharp contrast with many earlier studies where the net transfer of isotope from mevalonate into a given target compound

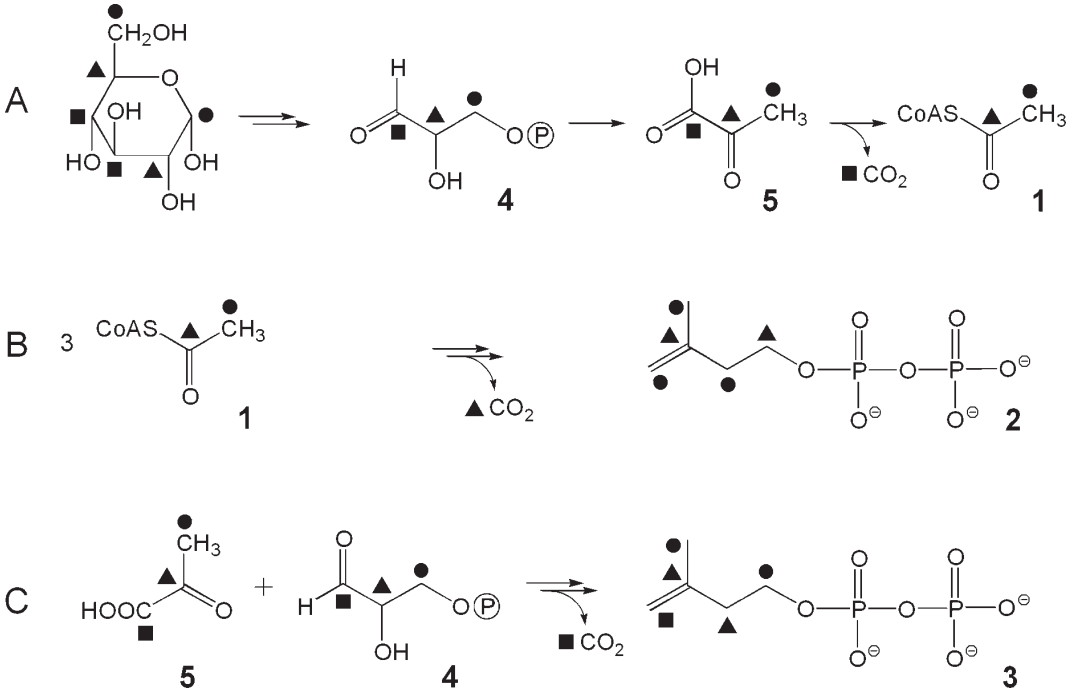

Figure 2. (*A*), Predicted labelling patterns of glyceraldehyde 3-phosphate (**4**), pyruvate (**5**) and acetyl-CoA (**1**) starting from isotope-labelled glucose via glycolysis followed by the pyruvate dehydrogenase reaction. (*B*), predicted labelling pattern of IPP (**2**) biosynthesized from acetyl-CoA (**1**) via mevalonate. (*C*), predicted labelling pattern of IPP (**2**) biosynthesized from pyruvate (**5**) and glyceraldehyde 3-phosphate (**4**) via 1-deoxy-D-xylulose 5-phosphate. The symbols indicate biosynthetically equivalent positions.

was taken as bona fide evidence for the mevalonate origin of the compound under study.

It is also obvious that the mevalonate pathway can at best transfer blocks of two labelled carbon atoms from [U-$^{13}C_6$]glucose (i.e. via [U-$^{13}C_2$]acetyl-CoA) to the target molecule, whereas a block of three labelled carbon atoms can be transferred by the deoxyxylulose pathway (i.e. via [$^{13}C_3$]glyceraldehyde 3-phosphate), albeit under bond breakage and fragment religation brought about by 2C-methylerythritol 4-phosphate synthase (IspC protein) (see below). NMR spectroscopy can diagnose the joint transfer of ^{13}C atom groups, even in case of an intramolecular rearrangement, by a detailed analysis of the ^{13}C coupling pattern via one- and two-dimensional experiments, and indeed, the NMR detection of $^{13}C_3$ fragments in plant terpenoids from experiments with [U-$^{13}C_6$]glucose was crucial for the discovery of the non-mevalonate pathway in plants [14, 29].

In a more rigorous approach, the ensemble of metabolic precursors in a given experimental system is treated as a network with hundreds to thousands of nodes where an isotope label can spread in every direction. If the isotope distribution in such a system is experimentally determined at a sufficient number of metabolic sinks (e.g. biosynthetic amino acids and nucleotides), the labelling patterns of central metabolites such as acetyl-CoA (**1**), pyruvate/hydroxyethyl-TPP (**8**) and glyceraldehyde 3-phosphate (**4**) can be reconstructed from the labelling patterns of their respective downstream products, such as leucine (**6**), valine (**7**) and tyrosine (**9**) [44–50] (fig. 3). These data can be used to construct hypothetical labelling patterns of secondary metabolites via different hypothetical pathways (e.g. via the mevalonate and non-mevalonate pathway). The predicted patterns are then compared with experimentally determined labelling patterns.

The biosynthesis of the diterpene **11** of the verrucosane family [34] can serve to illustrate the approach. The predicted labelling pattern *via* mevalonate (**10**) (fig. 4A) was at odds with the observed pattern of **11** after feeding of [1-^{13}C]glucose (fig. 4C), whereas the predicted pattern via 1-deoxyxylulose 5-phosphate (**12**) (fig. 4B) was in perfect agreement. It was therefore concluded that, in the liverwort *Fossombronia alaskana*, the biosynthesis of the polycyclic molecule **11** proceeds via the non-mevalonate pathway [34]. On the other hand, a structurally similar verrucosane-type diterpene is obtained *via* the mevalonate pathway in the bacterium *Chloroflexus aurantiacus* [51].

Incorporation studies with ^{13}C-labelled glucose are not limited to delineating the origin of the building blocks but

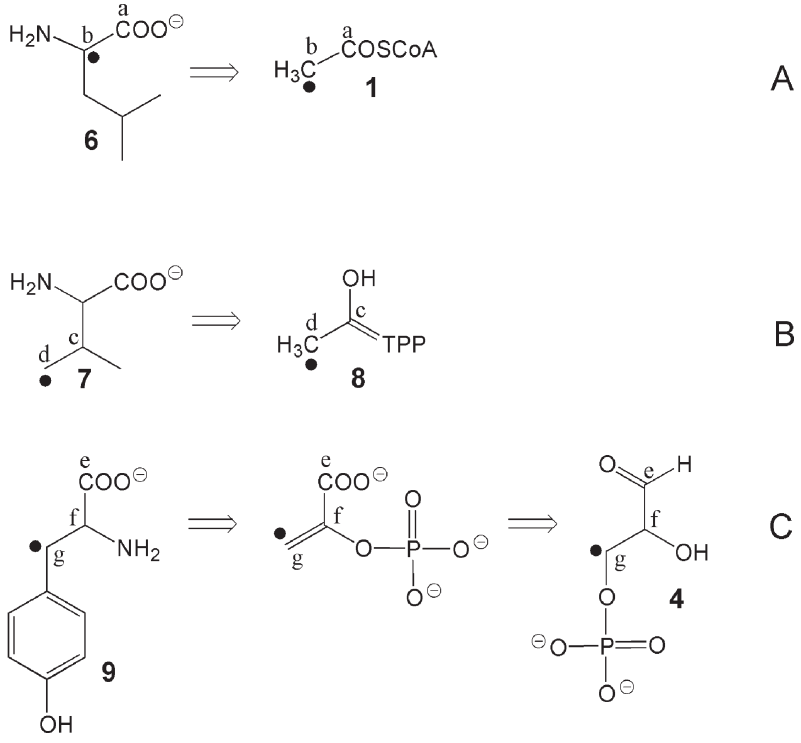

Figure 3. Retrobiosynthetic analysis of amino acids. (*A*), reconstruction of the labelling pattern of acetyl-CoA (**1**) from the labelling pattern of leucine (**6**). (*B*), reconstruction of the labelling pattern of hydroyethyl-TPP (**8**) from the labelling pattern of valine (**7**). (*C*), reconstruction of the labelling pattern of phosphoenolpyruvate and glyceraldehyde 3-phosphate (**4**) from the labelling pattern of tyrosine (**9**). Small characters indicate biosynthetically equivalent positions. The filled circles indicate ^{13}C-enriched carbon atoms from [1-^{13}C]glucose in the relevant moieties in plants and liverworts (cf. also fig. 2).

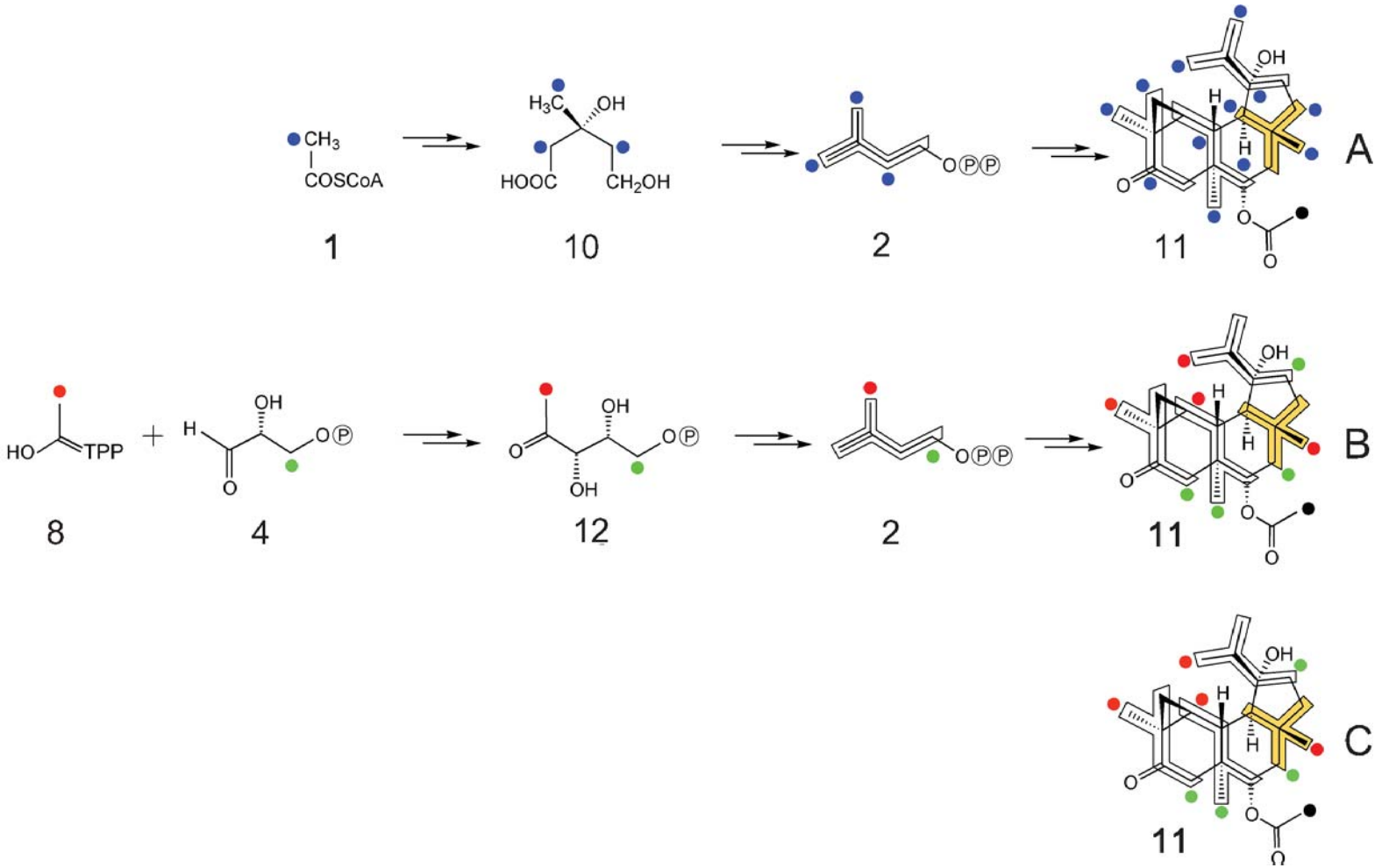

Figure 4. Observed and predicted labelling patterns of 8α-acetoxy-13α-hydroxy-5-oxo-13-*epi*-neoverrucosane (**11**) after growing the liverwort *Fossombronia alaskana* on a medium containing [1-^{13}C]glucose. Filled circles indicate carbon atoms ^{13}C-enriched from [1-^{13}C]glucose. (*A*), predicted labelling pattern (shown by blue-coloured circles) in **11** biosynthesized via [2-^{13}C]acetyl-CoA (**1**). (*B*), predicted labelling pattern in **11** biosynthesized via hydroxyethyl-TPP (**8**) (cf. red-coloured circles), glyceraldehyde 3-phosphate (**4**) (cf. green-coloured circles) and 1-deoxyxylulose 5-phosphate (**12**). (*C*), labelling pattern of **11** determined by quantitative NMR spectroscopy. The labelling patterns of **1**, **8** and **4** were reconstructed by retrobiosynthetic analysis of amino acids obtained from cellular protein in the same labelling experiments (cf. fig. 3). Carbon atoms contributed by individual C_5 monomers are boxed.

can also serve to dissect the complex reaction mechanisms for the formation of structurally complex terpenes. Since the biosynthesis of many terpenes involves one or more skeletal rearrangement(s), dissecting the isoprenoid building blocks affords important clues with regard to the downstream biosynthetic mechanism. In favourable cases, very complex reaction mechanisms can be extracted reliably from a small number of experiments. The mechanism for the formation of the diterpene **11** from the aliphatic precursor **13** is shown in figure 5 as a specific example where the labelling pattern of the biosynthetic product, **11,** indicated a skeletal rearrangement involving the C_5 unit coloured in yellow [34].

The biosynthetic origin of a considerable number of primary and secondary plant terpenoids has been reinvestigated recently using the technology described above. The experimental systems included members of the gymnosperm and angiosperm families of higher plants as well as liverworts as examples for lower plants. The data show that sterols are synthesized predominantly via the mevalonate pathway-derived precursors in all plants analysed [14, 28, 39, 43]. Ubiquinone is biosynthesized in plant mitochondria using mevalonate-derived precursors from the cytoplasm [52].

A wide variety of monoterpenes and diterpenes has been shown to be biosynthesized predominantly via the deoxyxylulose pathway (reviewed in [16]). They include compounds with fundamental physiological significance for all plants as well as a much larger number of compounds which are restricted to specific taxonomic groups.

Most notably, the phytol side chain which recruits chlorophylls, the most abundant organic pigments, to the thylakoid membrane, is predominantly formed via the deoxyxylulose pathway in higher plants. Carotenoids, which play a central role in all green plants as light-protecting and light-harvesting agents as well as specific roles as pigments in flowers, are derived from the deoxyxylulose pathway.

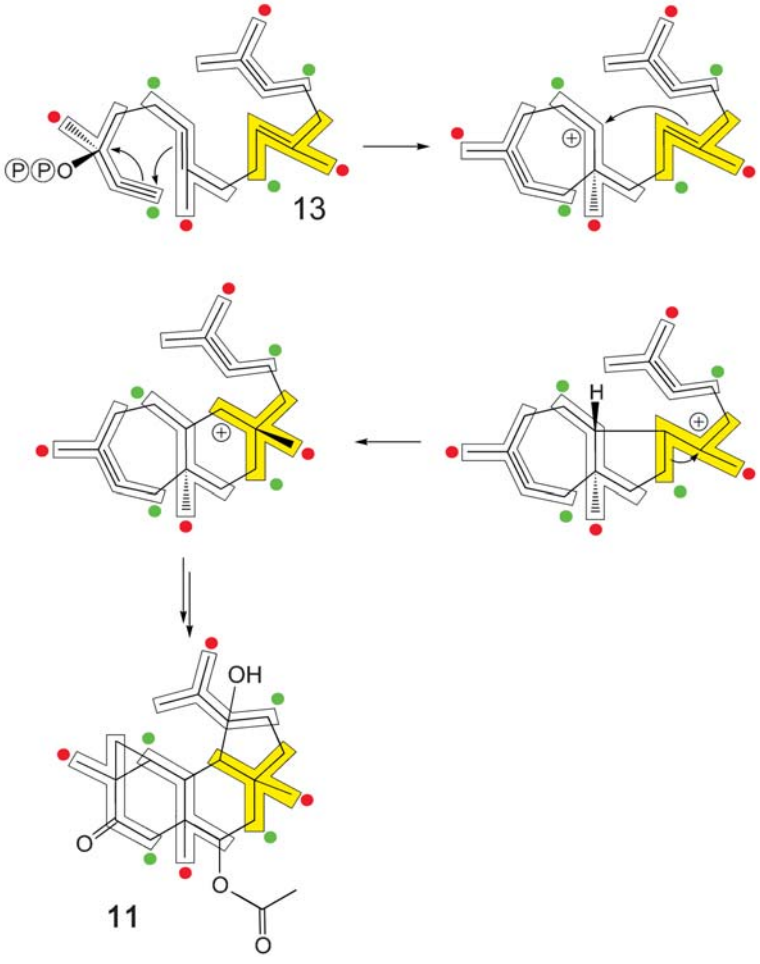

Figure 5. Hypothetical mechanism for the formation of 8α-acetoxy-13α-hydroxy-5-oxo-13-*epi*-neoverrucosane (**11**) from geranyllinaloyl diphosphate (**13**) derived via 1-deoxy-D-xylulose 5-phosphate in the liverwort *F. alaskana*. Carbon atoms contributed by individual C_5 monomers are boxed. The C_5 unit involved in a skeletal rearrangement is shown in yellow. Filled circles indicate carbon atoms ^{13}C-enriched from [1-^{13}C]glucose. The labelling pattern of **11** was determined by quantitative NMR spectroscopy (cf. also fig. 4).

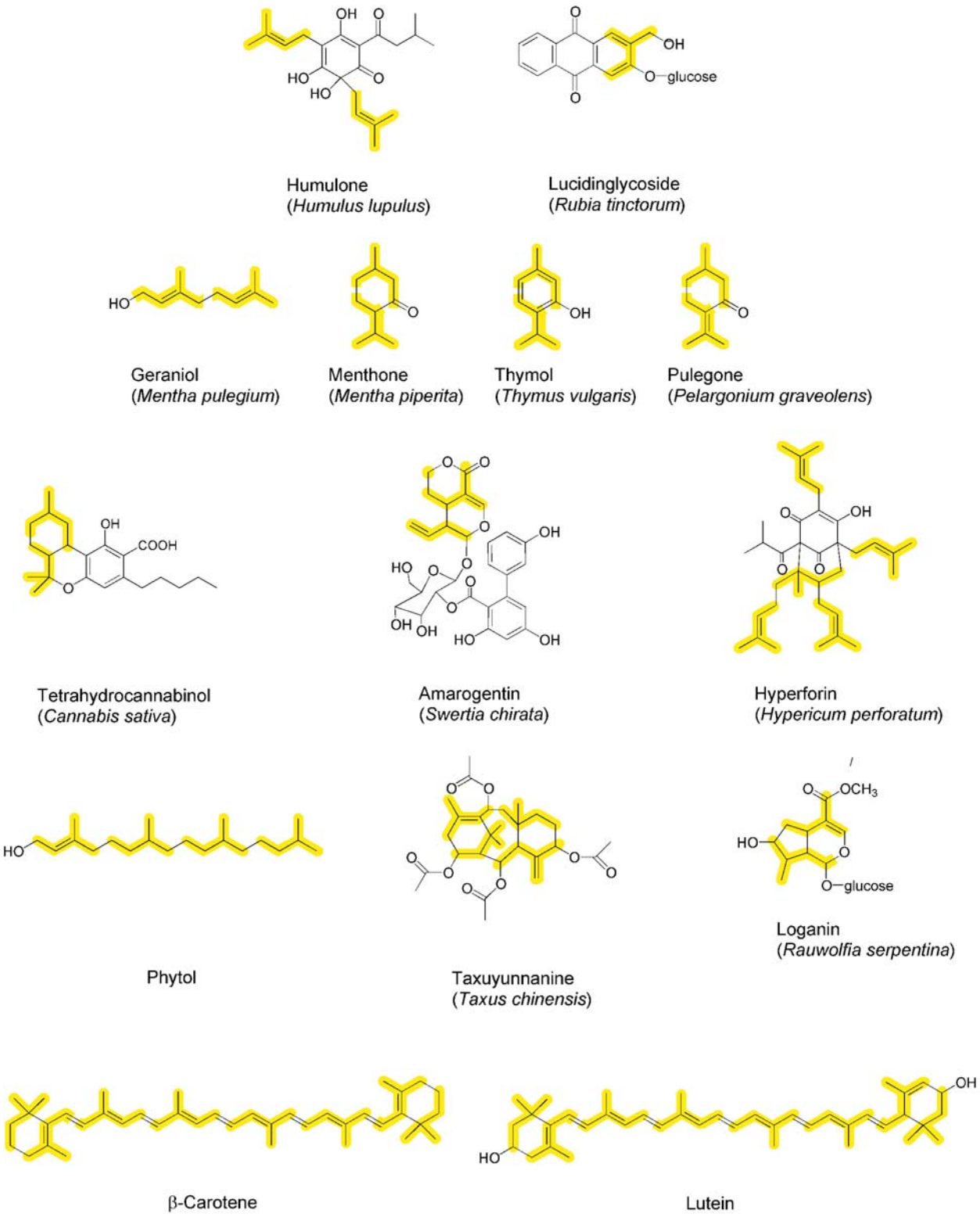

Figure 6. Examples of isoprenoids and meroterpenoids biosynthesized via the deoxyxylulose phosphate pathway. Carbon atoms contributed by individual C_5 monomers are highlighted in yellow.

Other representative examples of plant natural products derived entirely or partially from the deoxyxylulose pathway include taxoids from yew [29], iridoid alkaloids derived from loganin [37], essential oils [33], the terpenoid moieties of bitter acids from hops [38], anthraquinones [36], cannabinoids [41], the bitter-tasting amarogentin [40] and the anti-depressant hyperforin [42] (cf. fig. 6).
Surprisingly, certain terpenes are biosynthetic mosaics where one or several isoprenoid building blocks are derived from the chloroplast-based deoxyxylulose pathway, whereas others are derived from the cytoplasmic mevalonate pathway [30–32, 53, 54]. This implicates that certain biosynthetic steps proceed in different compartments, and that specific intermediates traverse the chloroplast boundary. The extent of this crosstalk between the different metabolic compartments can be modulated by feeding of specific precursors (1-deoxyxylulose respectively mevalonate) [27, 53].

Genes and enzymes of the non-mevalonate pathway

During the past decade, information on the alternative, non-mevalonate pathway has been unfolding rapidly, as summarized in figure 7. Briefly, 1-deoxy-D-xylulose 5-phosphate (**12**) obtained by condensation of pyruvate (**5**) and D-glyceraldehyde 3-phosphate (**4**) undergoes a rearrangement coupled to a reduction step. The resulting 2*C*-methyl-D-erythritol 4-phosphate (**14**) is converted into its cyclic diphosphate (**17**) by the sequential action of three enzyme activities. 2*C*-Methyl-D-erythritol 2,4-diphosphate (**17**) is transformed into IPP (**2**) and DMAPP (**3**) via 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**). These reaction steps are described in more detail below.

1-Deoxy-D-xylulose 5-phosphate synthase (Dxs protein) (EC 2.2.1.7)

The formation of the carbon skeleton of **12** by condensation of **4** with **5** is mechanistically reminiscent of the reaction catalysed by transketolases. Sequence similarity of an unannotated gene of *E. coli* (later named *dxs*) with genes encoding transketolases (EC 2.2.1.1) suggested that the cognate enzyme could catalyse the formation of **12** and carbon dioxide [55, 56]. This hypothesis was confirmed by expression of the gene in a recombinant *E. coli* strain [55, 56].
1-Deoxy-D-xylulose 5-phosphate synthase genes from a variety of plants and microorganisms have been cloned and expressed (cf. Table 1). The cognate enzymes require Mg^{2+} and thiamine diphosphate (**19**) which acts as carrier for an acyl anion synthon (fig. 8), in close parallel with the reaction mechanisms of transketolase (EC 2.2.1.1), of

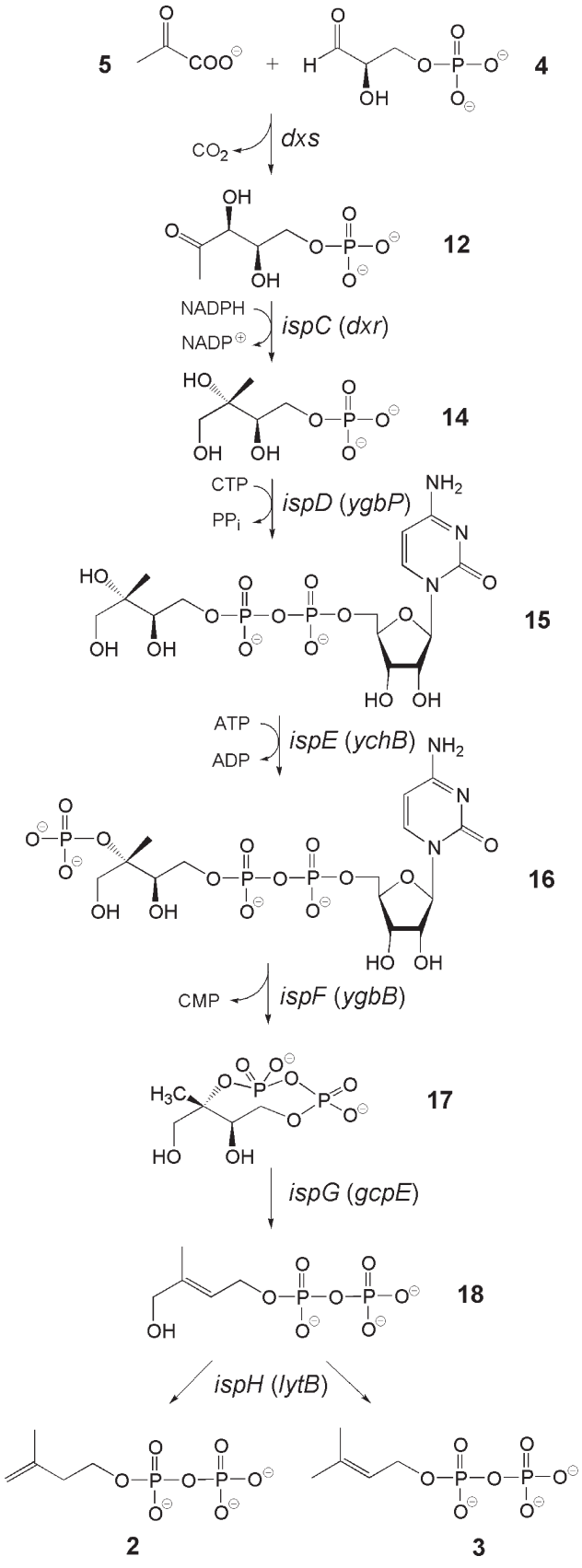

Figure 7. Deoxyxylulose phosphate pathway of IPP (**2**) and DMAPP (**3**) biosynthesis.

Table 1. Recombinant enzymes of the non-mevalonate pathway. The numbers indicate references as given in the reference list of this review.

Organisms	Characterized proteins						
	Dxs	IspC	IspD	IspE	IspF	IspG	IspH
Bacteria							
Aquifex aeolicus							130
Bacillus subtilis	57, 58						
Escherichia coli	55, 56	78-81	98, 100	105, 109	106, 111	122, 123	123, 129
Pseudomonas aeruginosa	59	59					
Rhodobacter capsulatus	60, 61						
Streptomyces coelicolor	62	62	62				
Streptomyces sp.	63						
Synechococcus leopoliensis	64	82					
Synechocystis sp.	58	83					
Thermus thermophilus						124	
Zymomonas mobilis		84					
Plants							
Arabidopsis thaliana	65–67	74, 85, 86	99			125	
Capsicum annuum	68						
Catharantus roseus	69						
Lycopersicon esculentum		87		108			
Mentha piperita	70	88		88			
Protozoa							
Plasmodium falciparum		89			112		

the E1 component of the pyruvate dehydrogenase complex (EC 1.2.4.1), of pyruvate decarboxylase (EC 4.1.1.1) and of acetolactate synthase (EC 4.1.3.18). The transketolase-like features of these proteins are in line with weak, but significant sequence similarities. In *Streptomyces* sp. and in *Rhodobacter capsulatus* as well as in certain plants, the existence of multiple *dxs* orthologs has been reported [60, 71, 72]. The *dxs* genes from plants specify putative plastid-targeting sequences, in line with the plastid localization of the deoxyxylulose phosphate pathway in plants.

The catalytic activities of 1-deoxy-D-xylulose 5-phosphate synthases are relatively high (300–500 µmol mg^{-1} min^{-1}) by comparison with the following enzymes of the pathway (see below). Steady-state kinetic studies and trapping experiments with ^{14}C-labelled substrate indicated a ping-pong mechanism in which pyruvate (**5**) binds first, CO_2 is released, D-glyceraldehyde 3-phosphate (**4**) binds next and, finally, 1-deoxy-D-xylulose 5-phosphate (**12**) is released from the enzyme [61]. An essential role of residue H-49 in the catalytic process was demonstrated for the *E. coli* enzyme [73]. Indeed, H-49 is highly conserved in orthologs of the *E. coli* enzyme, as well in transketolases. The enzyme from *Chlamydomonas* has been shown to be weakly inhibited by ketochlomazone [74].

Besides D-glyceraldehyde 3-phosphate, 1-deoxy-D-xylulose 5-phosphate synthase from *E. coli* can use other sugar phosphates as well as short-chain aldehydes as acceptor substrates; apart from pyruvate, hydroxypyruvate and 2-oxobutyrate are accepted [75].

In *Arabidopsis thaliana* and tomato, the levels of several plastidic isoprenoids are correlated with changes in 1-deoxy-D-xylulose 5-phosphate synthase levels. It was therefore proposed that 1-deoxy-D-xylulose 5-phosphate synthase is the rate-limiting enzyme for IPP/DMAPP biosynthesis in plants [61, 76].

2*C*-Methyl-D-erythritol 4-phosphate synthase (IspC protein, Dxr protein) (EC 1.1.1.267)

The generation of the branched chain isoprenoid precursors from the linear carbon skeleton of **12** requires a skeletal rearrangement as documented by in vivo studies on ginkgolides [14], taxoids [29], menaquinone [77], carotenoids and phytol [25]. In summary, these data showed unequivocally that carbon atom 4 of **12** becomes connected to C-2 by an intramolecular process (fig. 9A).

An enzyme specified by the *ispC* (formerly designated *yaeM* or *dxr*) gene catalysing the conversion of **12** into **14** was first isolated from *E. coli* [78]. Homologous proteins were subsequently expressed from various bacteria, plants and the protozoon *Plasmodium falciparum* (cf. table 1 and references therein). The catalytic rates of the 2*C*-methyl-D-erythritol 4-phosphate synthases are substantially lower compared with those of 1-deoxy-D-xylulose 5-phosphate synthases, in the range of 12 µmol mg^{-1} min^{-1}. The enzymes can use Mg^{2+} or Mn^{2+} as cofactor. At low concentrations, Mn^{2+} is more efficient than Mg^{2+}.

The enzyme-catalysed reaction is initiated by the skeletal rearrangement which is followed by a two-electron re-

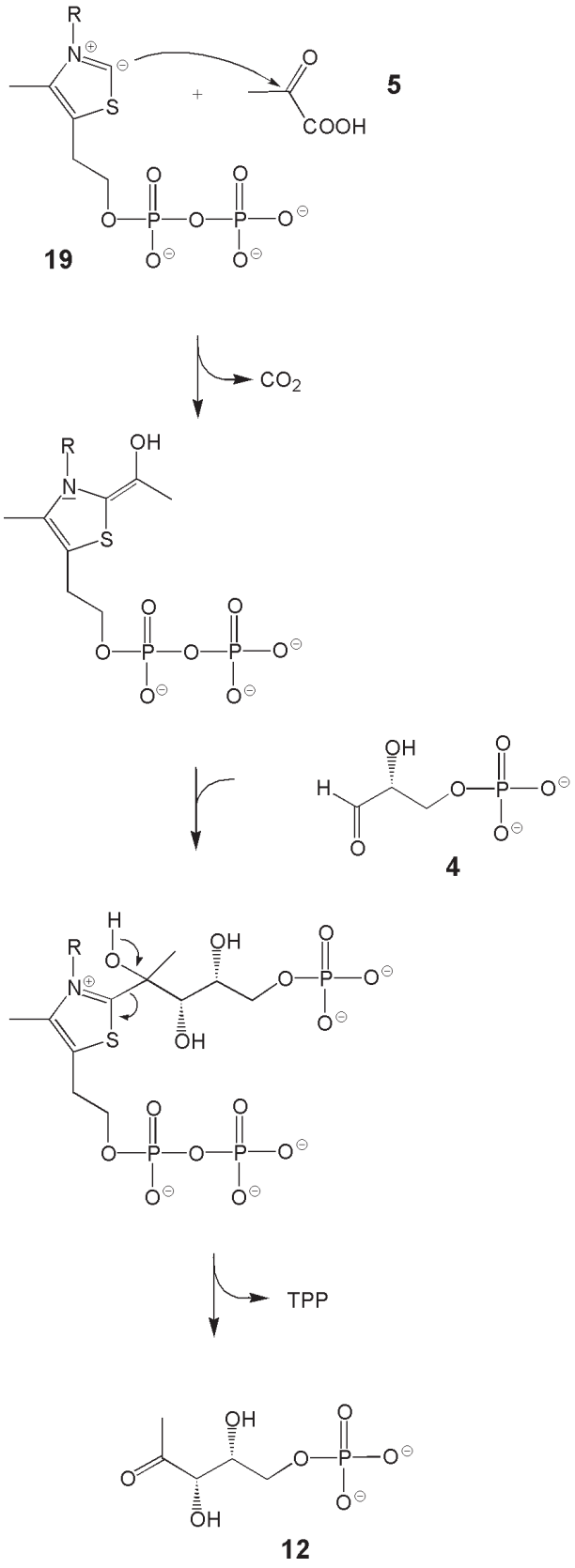

Figure 8. Proposed mechanism for the formation of 1-deoxyxylulose 5-phosphate (**12**) catalysed by 1-deoxyxylulose 5-phosphate synthase (DxS protein). TPP, thiamine pyrophosphate.

duction step requiring NADPH (**21**) as cofactor (fig. 9A) and can be inhibited by fosmidomycin (**22**, fig. 9B) at nanomolar concentrations [79, 89–91]. A reaction intermediate, 2*C*-methyl-D-erythrose 4-phosphate (**20**), has been identified, and solid evidence has been presented for the reversibility of the reaction [79, 80]. The formation of **14** is thermodynamically favoured under physiological conditions. The forward reaction proceeds as an ordered process with NADPH binding prior to 1-deoxy-D-xylulose 5-phosphate [79, 80].

Studies on the biosynthesis of 2*C*-methyl-D-erythritol in leaves of the tulip tree implied hydride addition to the RE face of **20** [92]. The stereochemical features of the formation of 2*C*-methyl-D-erythritol 4-phosphate were also studied with ^{2}H-labelled substrates using the recombinant 2*C*-methyl-D-erythritol 4-phosphate synthases from *E. coli* [81] and *Synechocystis* sp. PCC6803 [83]. NMR analysis showed that both enzymes catalyse the stereospecific transfer of H_{Si} from C-4 of NADPH (**21**) to the RE face of the aldehyde-type intermediate (**20**) (fig. 9A).

The structure of the *E. coli* enzyme was determined by X-ray crystallography [93–95]. Each subunit of the homodimer consists of an N-terminal dinucleotide binding domain, a connecting domain with the catalytic site and a C-terminal helical domain. The structure of a complex with NADPH [94] confirmed the essential role of Gly-14, Glu-231, His-153, His-209 and His-257 in the catalytic process [96]. The crystal structure also validates an earlier NMR study in which intermolecular nuclear Overhauser effects (NOEs) indicated that the methyl group of a methionine residue (Met-214) of the protein is located close to the nicotinamide ring portion of the NADPH substrate [97]. In the crystal structure of the *E. coli* protein complexed with Mn^{2+} and the inhibitor fosmidomycin (**22**, fig. 9B), the substrate molecule **12** can be superimposed onto fosmidomycin, indicating that fosmidomycin binds in a substrate-like mode [95]. The detailed knowledge of the interaction mode of fosmidomycin should benefit the development of more potent inhibitors.

4-Diphosphocytidyl-2*C*-methyl-D-erythritol 4-phosphate synthase (IspD protein) (EC 2.7.7.60)

Complete genomes of numerous bacterial and several eukaryotic species have been reported recently and represent a treasure trove for the study of metabolism. Several genes of the non-mevalonate pathway downstream from *ispC* were discovered by a strategy combining biochemical evidence with comparative genomic analysis, as described below.

As a first step in the search for subsequent enzymes and intermediates of the pathway, crude cell extracts of *E. coli* were shown to form unidentified products from **14** in the presence of nucleoside triphosphates, preferentially cyti-

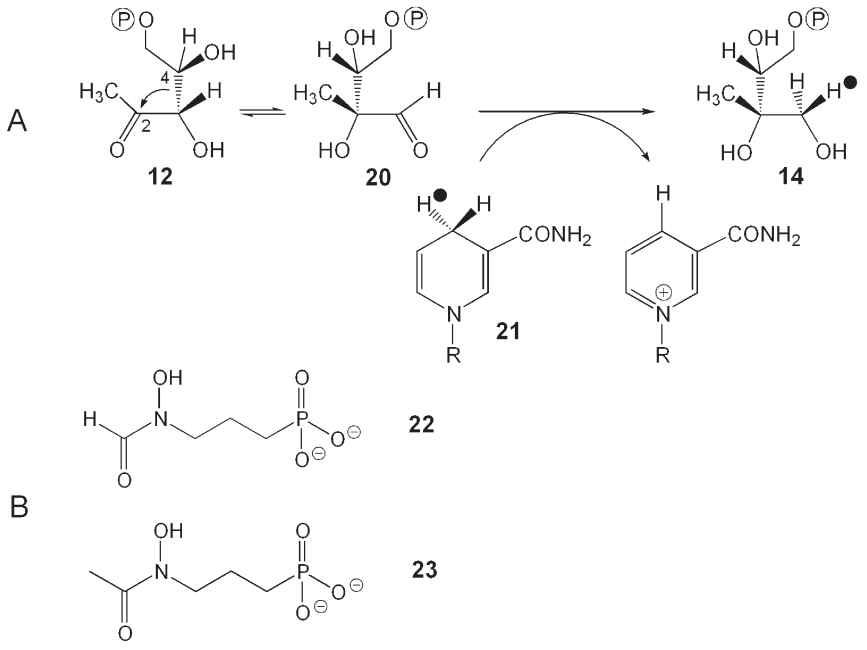

Figure 9. (*A*) Stereochemical course of the reaction catalysed by IspC protein. (*B*), structures of the inhibitors fosmidomycin (**22**) and FR-900098 (**23**).

dine triphosphate (CTP), thus suggesting the transfer of a nucleoside phosphate moiety to **14** [98]. A database search for phosphocytidyl transferases retrieved the *acs1* gene of *Haemophilus influenzae* [98]. The N-terminal domain of the cognate enzyme catalyses the transfer of a cytidine monophosphate (CMP) moiety to ribitol 5-phosphate affording 5-diphosphocytidyl ribitol. An extended sequence similarity search using the phosphocytidyl transferase moiety of the *acs1* gene as search motif retrieved the unannotated *E. coli* gene *ygbP* (later renamed *ispD*). This gene was expressed in a recombinant bacterial strain, and the cognate enzyme was shown to catalyse the transfer of a phosphocytidyl moiety to **14** under formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol (**15**) and inorganic pyrophosphate [98] (cf. fig. 7).

4-Diphosphocytidyl-2*C*-methyl-D-erythritol synthases were also cloned from *Streptomyces coelicor* [62] and *A. thaliana* [99]. Besides Mg^{2+}, the *E. coli* enzyme can use Mn^{2+} and Co^{2+} ions as cofactor, whereas the plant enzyme is also catalytically active in the presence of Ni^{2+} ions. The catalytic activity of all orthologs studied up to now (table 1) is relatively low (20–70 µmol min^{-1} mg^{-1}) compared with the first pathway enzyme, 1-deoxy-D-xylulose 5-phosphate synthase. The second substrate, CTP, can be replaced by other nucleotide triphosphates, albeit at low reaction rates [98]. *D*-Erythritol 4-phosphate inhibits the IspD protein of *E. coli* with a concentration of the inhibitor that produced half maximal effect (IC_{50}) of 1.4 mM [101, 102].

The structure of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase from *E. coli* has been determined by X-ray crystallography. The enzyme is a homodimer. In the enzyme substrate complex, Mg^{2+} is coordinated to the α, β and γ phosphate residues of CTP but not to the protein [103, 104].

Further genomic analysis including all completely sequenced genomes in the public domain showed that the distribution of the *ispD* gene parallels the occurrence of putative orthologs of the *ispC* gene [98] (table 2). Moreover, this distribution was well in line with available biochemical data on the occurrence of the mevalonate and non-mevalonate biosynthetic pathways which had been derived from comparative isotope incorporation studies (see below).

In the genome of *E. coli* and several other eubacteria, the *ispD* gene was found to be closely linked to the unannotated *ygbB* (now *ispF*) gene or its putative orthologs. Moreover, several bacteria (e. g. *Helicobacter pylori*) were found to specify putative bifunctional fusion genes comprising *ispD* and *ispF* domains [98]. The distribution of genes with similarity to the *ispF* gene in completely sequenced genomes mimicked the distribution of the *dxs*, *ispC* and *ispD* genes (table 2).

A systematic search for additional orthologous groups matching the distribution of the genes described above retrieved the *ychB (ispE), lytB (ispH)* and *gcpE* (*ispG)* genes [105–107] (table 2). They were all subsequently confirmed to be involved in the non-mevalonate pathway.

Table 2. IPP and DMAPP biosynthesis genes in representative examples of completely sequenced organisms.

Organisms	Deoxyxylulose phosphate pathway							Mevalonate pathway						
	dxs	ispC	ispD	ispE	ispF	ispG	ispH	hmgs	hmgr	mk	pmk	dpmd	idiI	idiII
Bacteria														
Aquifales (*Aquifex aeolicus*)	+	+	+	+	+	+	+	–	–	–	–	–	–	–
Chlamydia group (*Chlamydophila pneumoniae*)	+	+	+	+	+	+	+	–	–	–	–	–	–	–
Cyanobacteria (*Synechocystis* sp.)	+	+	+	+	+	+	+	–	–	–	–	–	–	+
Deinococcus group (*Deinococcus radiodurans*)	+	+	+	+	+	+	+	–	–	–	–	–	–	+
Firmicutes (*Bacillus subtilis*)	+	+	+	+	+	+	+	–	–	–	–	–	–	+
(*Mycoplasma genitalium*)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(*Staphylococcus aureus*)	–	–	–	–	–	–	–	+	+	+	+	+	–	+
(*Streptomyces coelicolor*)	+	+	+	+	+	+	+	–	–	–	–	–	+	–
(*Listeria monozytogenes*)	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Proteobacteria (*Escherichia coli*)	+	+	+	+	+	+	+	–	–	–	–	–	+	–
(*Rickettsia prowazeckii*)	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Spirochaetales (*Treponema pallidum*)	+	+	+	+	+	+	+	–	–	–	–	–	–	–
(*Borrelia burgdorferi*)	–	–	–	–	–	–	–	+	+	+	+	+	–	+
Thermotogales (*Thermotoga maritima*)	+	+	+	+	+	+	+	–	–	–	–	–	–	–
Archaea														
Crenarchaeota (*Aeropyrum pernix*)	–	–	–	–	–	–	–	+	+	+	–	–	–	+
Euryarchaeota (*Archaeoglobus fulgidus*)	–	–	–	–	–	–	–	+	+	+	–	–	–	+
Eukaryotes														
Animals (*Homo sapiens*)	–	–	–	–	–	–	–	+	+	+	+	+	+	–
Plants (*Arabidopsis thaliana*)	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Plasmodium falciparum	+	+	+	+	+	+	+	–	–	–	–	–	–	–
Yeasts (*Saccharomyces cerevisiae*)	–	–	–	–	–	–	–	+	+	+	+	+	+	-

Abbreviations: *dxs*, 1-deoxy-D-xylulose 5-phosphate synthase; *ispC*, 2*C*-methyl-D-erythritol 4-phosphate synthase; *ispD*, 4-diphosphocytidyl 2*C*-methyl-D-erythritol synthase; *ispE*, 4-diphosphocytidyl 2*C*-methyl-D-erythritol kinase; *ispF*, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *ispG*, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; *ispH*, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; *hmgs*, 3-hydroxy-3-methylglutaryl-CoA synthase; *hmgr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *mk*, mevalonate kinase; *pmk*, phospomevalonate kinase; *dpmd*, diphospomevalonate decarboxylase; *idiI*, isopentyldiphosphate isomerase type I, *idiII*, isopentyldiphosphate isomerase type II.

4-Diphosphocytidyl-2*C*-methyl-D-erythritol kinase (IspE protein) (EC 2.7.1.148)

The recombinant IspE protein of *E. coli* was shown to phosphorylate the position 2 hydroxy group of **15** under formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (**16**; cf. fig. 7) [105]. The enzyme requires adenosine triphosphate (ATP) as second substrate and Mg^{2+} as cofactor. Sequence comparison revealed similarities to other ATP-dependent kinases such as mevalonate kinase, homoserine kinase and phosphomevalonate kinase. The orthologous gene from tomato (*Lycopersicum esculentum*) has also been cloned and expressed [108] (table 1).The recombinant IspE protein from *Mentha piperita* was claimed to convert isopentenyl monophosphate into IPP [88]. However, in light of the small reaction rates that have been reported (86 fmol mg^{-1} min^{-1}), the phosphorylation of isopentenyl monophosphate by IspD has no physiological relevance.

The structure of a ternary complex of the homodimeric 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase from *E. coli* with **15** and an analogue of ATP has been determined by X-ray crystallography [110]. The enzyme subunits display an α/β-fold characteristic of the GHMP kinase superfamily. The catalytic center is located in a deep cleft between protein domains and appears to be well suited for the structure-based design of inhibitors.

2*C*-Methyl-D-erythritol 2,4-diphosphate synthase (IspF protein) (E.C. 4.6.1.12)

The product of IspE protein, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate (**16**), is converted into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**17**) under release of CMP by the catalytic action of IspF protein (fig. 10A) [106, 111]. The protein can also convert **15** into 2*C*-methyl-D-erythritol 3,4-cyclophosphate (**24**), but the reaction rate is lower compared with the formation of **17** (fig. 10B) [106]. More recently, the orthologous IspF protein of *Plasmodium falciparum* was also shown to generate the cyclic monophosphate **24** from **15**, but the reaction velocity was modest compared with the formation of **17** [112, 113]. The enzymes require Mn^{2+} or Mg^{2+} as cofactor.

The structures of the IspF proteins from *E. coli*, *H. influenzae* and *Thermus thermophilus* have been determined by X-ray crystallography [114–118]. The three topologically equivalent active sites of the homotrimeric proteins contain Zn^{2+} ions coordinated by two conserved histidine and one aspartate residue [114]. The structures of several substrate and product complexes indicate that the zinc ion helps to position the substrate at the active site and facilitates the nucleophilic attack of the 2-phosphate group. The Mg^{2+} ion at the active site coordinates the α- and β-phosphate groups of the cytidine diphosphate (CDP) moiety.

2*C*-Methyl-D-erythritol 2,4-cyclodiphosphate reductase (IspG protein)

The role of the *ispG* (*gcpE*) gene was analysed by in vivo experiments with recombinant *E. coli* strains engineered for hyperexpression of non-mevalonate pathway genes in conjunction with the *xylB* gene specifying D-xylulokinase, which can catalyse the phosphorylation of 1-deoxy-D-xylulose [119] . A recombinant strain hyperexpressing *xylB* was shown to transform exogenous ^{13}C-labelled 1-deoxy-D-xylulose into its 5-phosphate (**12**) [120]. A recombinant strain hyperexpressing *xylB* together with *ispC,*

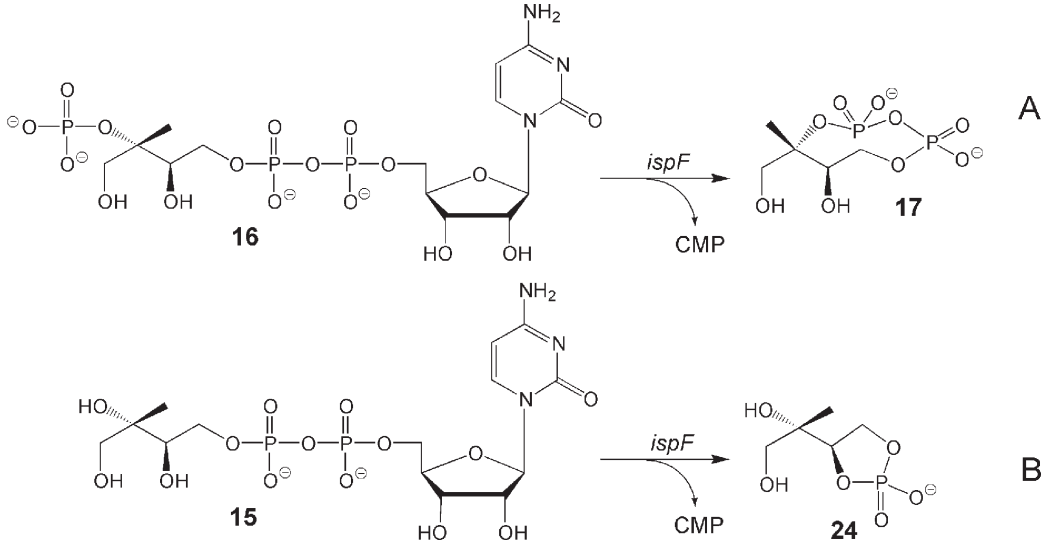

Figure 10. Reactions catalysed by IspF proteins from *E. coli* and *P. falciparum*.

ispD, ispE and ispF transformed ^{13}C-labelled 1-deoxy-D-xylulose into the cyclic diphosphate **17**. The additional implementation of *ispG* afforded an *E. coli* strain which transformed exogenous 1-deoxy-D-xylulose into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**) [120]. This finding indicated the involvement of the *ispG* translation product in the conversion of **17** into **18** (cf. fig. 7), which was also shown to accumulate in a mutant of *E. coli* carrying a deletion of the *ispH (lytB)* gene [121].

In vitro, recombinant IspG protein from *E. coli* catalyses the reductive ring opening of **17** using a photoreduced deazaflavin derivative as an artificial electron donor [122, 123]. Reduction equivalents for that reaction could also be supplied by NADPH using flavodoxin reductase and flavodoxin as a shuttle system for redox equivalents [122]. Under anaerobic conditions, the reduction of **17** catalysed by IspG protein of *T. thermophilus* could be driven by dithionite as reductant [124].

The absorption spectrum of purified IspG proteins from *E. coli* and *T. thermophilus* suggested the presence of [4Fe-4S]$^{2+}$ clusters [122–124]. Three absolutely conserved cysteine residues are believed to be part of these cofactors which are assumed to be essential for catalysis. A hypothetical radical mechanism is shown in figure 11, in which the epoxide **25** derived from **17** by hydroxyl-assisted solvolysis of the cyclopyrophosphate ring acts as a possible substrate for the reductive process. An additional bonus of this scheme is that the geometry of the epoxide intermediate **25**, dictated by the configuration of the two chiral centres in the precursor, is closely related to that of the final product, thus providing a welcome chemical rationale for the exclusive formation of the (*E*)-isomer of **18**.

The experimental systems described above have been reported to catalyse the formation of **18** at rates between 1–600 nmol mg^{-1} min^{-1} [122–124]. Notably, IspG protein catalysed that reaction more efficiently with the artificial electron donor system comprising dithionite compared with flavodoxin/flavodoxin reductase as a redox shuttle. Thus, additional work on the physiological electron donor system appears to be required.

1-Hydroxy-2-methyl-butenyl 4-diphosphate reductase (IspH protein)

The conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**) into IPP (**2**) and DMAPP (**3**) involves the translation product of the *ispH* (*lytB)* gene, which had been shown earlier to be essential in *E. coli* and *Synechocystis* sp. [121, 126].

In parallel to the in vivo experiments described for the *ispG* function, an *E. coli* strain engineered for expression of the genes *xylB* and *ispCDEFGH* converted exogenous ^{13}C-labelled 1-deoxy-D-xylulose into IPP and DMAPP at a molar ratio of 6:1 [127]. In addition, purified recombinant IspH protein from *E. coli*, by itself inactive, could be activated by crude cell extract from *E. coli,* catalysing the conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**) into **2** and **3** [128]. The observed molar ratio of **2** and **3** was similar to that detected in the in vivo

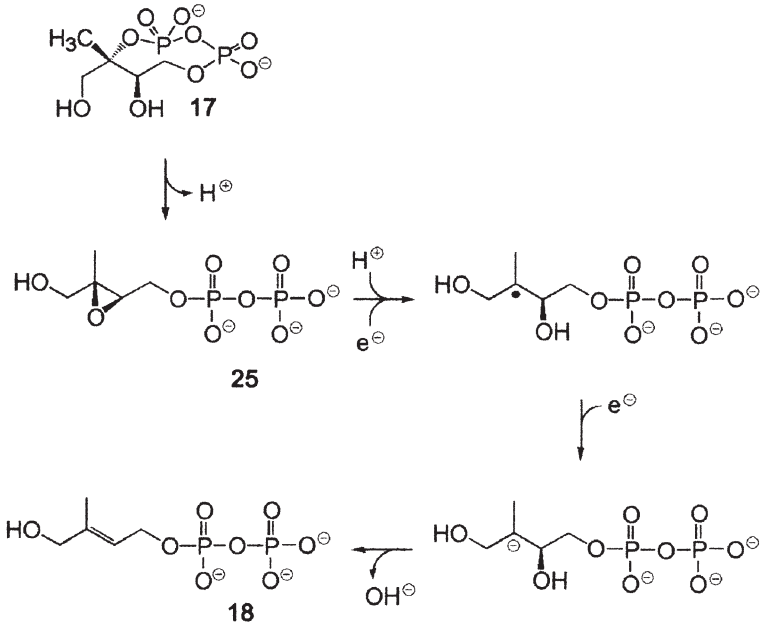

Figure 11. Hypothetical mechanism for the conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**17**) into 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate **(18**) catalysed by the IspG protein.

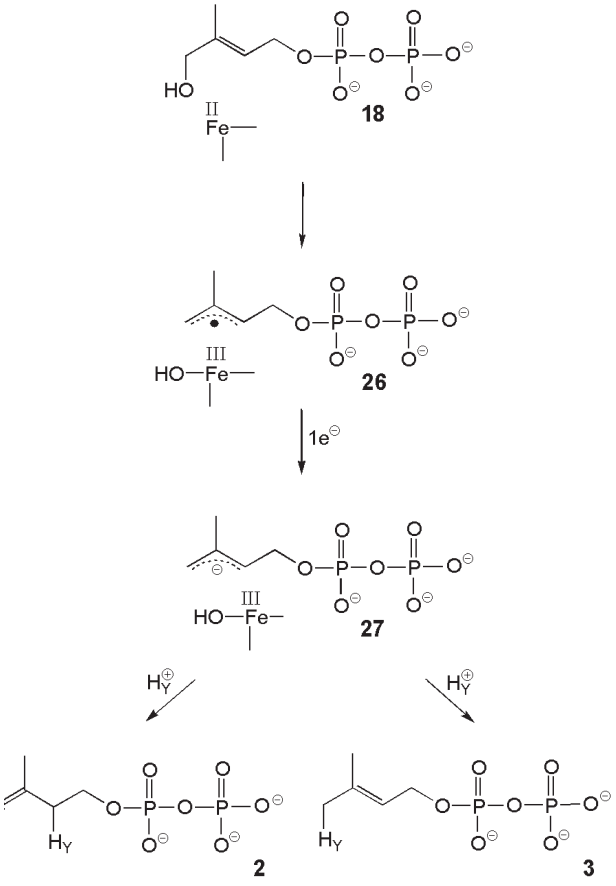

Figure 12. Hypothetical mechanism for the conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate **(18)** into IPP (**2**) and DMAPP (**3**) catalysed by the IspH protein.

experiments. The reaction required NADH, flavin-adenine dinucleotide (FAD) and divalent cations, preferably Co^{2+}, and probably at least one unidentified protein serving as electron shuttle. Activation of purified IspH protein from *E. coli* could also be achieved, under anaerobic conditions, by photoreduced 10-methyl-5-deaza-isoalloazine or by a mixture of flavodoxin, flavodoxin reductase and NADPH [123, 129]. Formation of **2** and **3** from **18** was also observed at a rate of 6.6 µmol mg^{-1} min^{-1} using IspH protein of *Aquifex aeolicus* as catalyst and dithionite as the reducing agent under anaerobic conditions [130].

Photometric analysis of recombinant IspH proteins from *E. coli* [123, 129] and from *A. aeolicus* [130], as well as electron paramagnetic resonance (EPR) studies on the *E. coli* enzyme [129] suggested the presence of a [4Fe-4S]$^{2+}$ cluster.

The reaction catalysed by the IspH protein involves the overall transfer of two electron equivalents, resulting in the cleavage of a nonactivated C-OH bond. As described above for the IspG reaction, the presence of a redox-active [4Fe-4S]$^{2+}$ cluster suggests the involvement of radical intermediates. On this basis, a hypothetical radical mechanism (fig. 12) was proposed via the allylic radical intermediate **26** and the allylic anion intermediate **27** [123].

Origin and fate of the carbon-bound H-ligands of IPP and DMAPP generated via the non-mevalonate pathway

Figure 13 presents an overview of the structural and stereochemical information which has been assembled in the last decade in studies of the new biosynthetic pathway to IPP and DMAPP. The methyl group of IPP (**2a**) as well as the (*Z*)-methyl group of DMAPP (**3a**) stem specifically from the methyl group of 1-deoxy-D-xylulose 5-phosphate (**12**) (Me in all the formulae of fig. 13). Early work carried out before the discovery of the new pathway had already indicated that upon feeding of [3-$^2H_3$]lactate to *E. coli*, in spite of extensive exchange of the label with solvent protons occurring most probably at the pyruvate level, some molecules managed to retain the original deuterium content during incorporation into terpenes [131]. Full retention of the three hydrogen atoms was eventually confirmed in experiments with appropriately labelled samples of the non-phosphorylated form of **12** [132] and **14** [133]. An occasional minor scrambling of a ^{13}C label between the methyl groups of DMAPP as well as between the methyl and the olefinic methylene group of IPP has been observed and blamed on the notorious lack of fidelity of the isomerase which interconverts the two compounds [113].

Intact transfer into the C-1 methylene groups of IPP and DMAPP has also been demonstrated for methylene groups of the free alcohols corresponding to **12** [15] and **14** [133]. Capitalizing on the fact that the origin of these methylene groups can be traced back to the methylene group of glyceraldehyde 3-phosphate (**4**), it has been shown that in *G. biloba* embryos, samples of **2** and **3** labelled stereospecifically with deuterium in their H_D-position are easily generated in vivo from [1-^{2}H]glucose via the operation of the glycolytic pathway [18].

Both the (*E*)-methyl group of DMAPP and the olefinic methylene group of IPP are derived from C-3 of 1-deoxy-D-xylulose 5-phosphate (**12**) via the hydroxymethyl group of 2*C*-methyl-D-erythritol 4-phosphate (**14**). Relevant stereochemical details concerning the formation of the latter have already been discussed in a previous section. Knowledge of the specific origin of the H_A- and H_X-ligands of **14** have played a crucial role for the understanding of the residual stereochemical signature detected in samples of phytol and lutein isolated from *Catharanthus* cell cultures after feeding of [2-^{13}C,3-^{2}H]1-deoxy-D-xylulose [134]. In connection with the known stereochemical course of the repetitive elongation process responsible for the formation of higher terpenes [135], these results provided indirect but nonetheless unequivocal evidence for the specific origin of the diastereotopically related olefinic

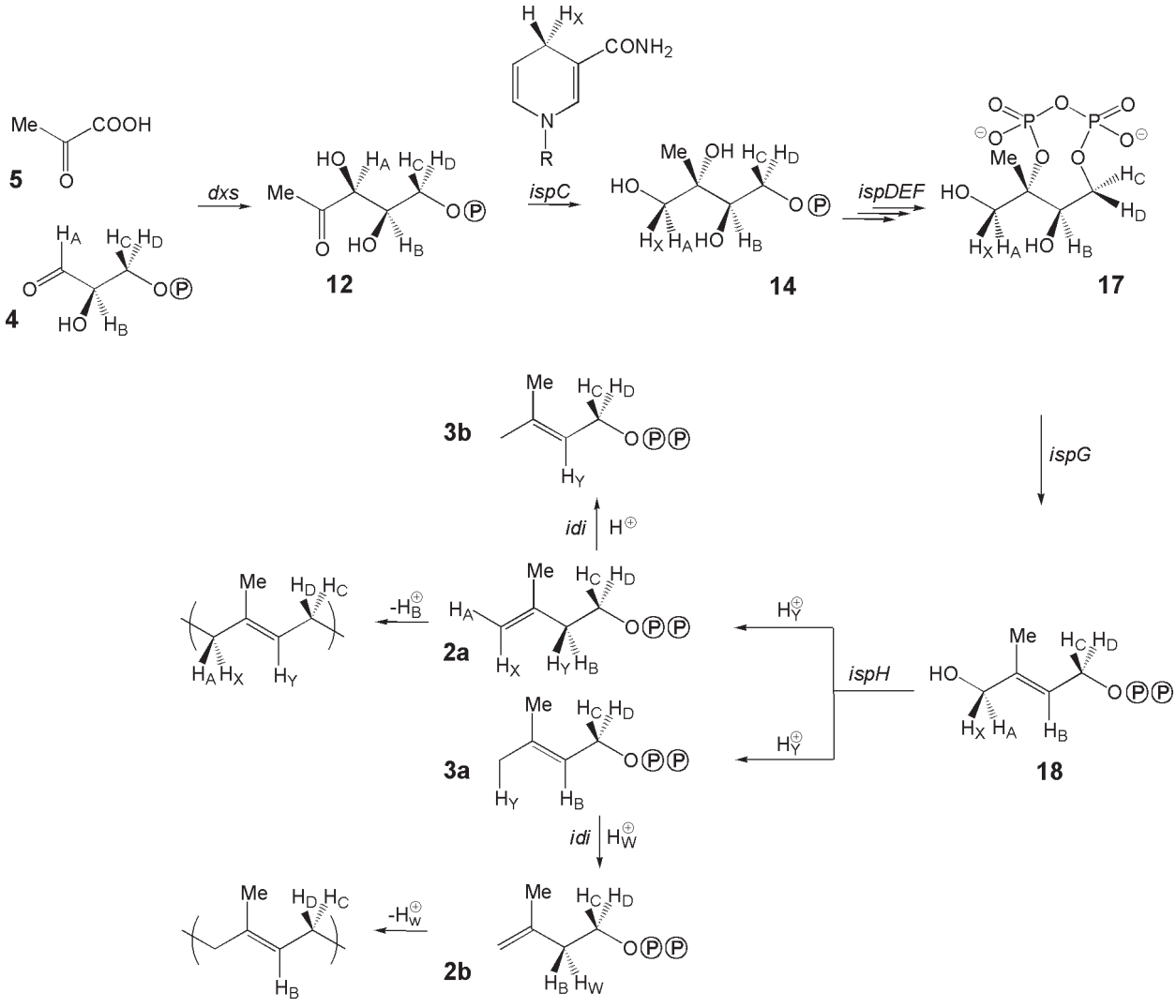

Figure 13. Overview of the stereochemical features of IPP and DMAPP biosynthesis via 1-deoxy-D-xylulose 5-phosphate (**12**). The origin of the carbon-bound H-atoms of IPP/DMAPP and higher terpenoids is indicated by capital characters.

hydrogens of **2a** indicated in figure 13. This, in turn, defines the half-space in which the hydroxy group of **18** is departing on the way to the ambident allylic anion **27** (cf. fig. 12) postulated as the branching point responsible for the parallel formation of **2a** and **3a**.

In both branches of this scheme, the new H-ligand (H_Y in fig. 13) is expected to be introduced as a proton; so far, there is no experimental basis for deciding whether a single proton or a pair of protons, possibly of different origins, are involved. In vitro experiments with extracts of *E. coli* have demonstrated that the electron input for the reduction of **18** is sustained by a set of not yet identified auxiliary proteins, requiring among others the participation of NADH and FAD [123, 128, 129]. Under these circumstances it is conceivable that the critical proton is generated in situ from $FADH_2$ (resulting from the NADH-dependent reduction of FAD) after transfer of the first electron to the substrate and manages, at least in part, to escape equilibration with the bulk water of the system prior to its quenching of the anionic intermediate formed in the second (and rapid) electron transfer (cf. fig. 12). Evidence compatible with such a possibility has been obtained in studies on the biosynthesis of hopanoid triterpenes in *Zymomonas mobilis*, which disclosed that the label from [1-^{2}H]glucose was transferred efficiently and most probably via [4-^{2}H]NADPH to all olefinic positions of the squalene intermediate [136]. No such transfer of label was observed, however, after application of the same precursor to embryos of *G. biloba* [18] or *Synechocystis* [137], probably as a consequence of the fact that these two organisms, in contrast to *Z. mobilis*, are endowed with the glycolytic pathway.

The specific origin of the enantiotopically related H-ligands at C-2 of IPP (**2a**) can be reconstructed as indicated

in figure 13 by combining the results of two independent investigations, namely (i) the demonstration that in *E. coli* the prenyltransferase engages specifically the (C-2)-H_{Re} of IPP [135] and (ii) the observation that in the same organism during terpene biosynthesis a deuterium label stemming from the H_B position of either **12** [138] or **14** [133] is retained in the DMAPP starter unit but not in the C_5-units derived from **2a** in the elongation step. While the cryptic stereochemical course of the reaction branch leading to **3a** remains veiled, the process which generates **2a** is now clearly revealed as a suprafacial one, a feature which may turn out to be relevant for the detailed mechanistic understanding of this unprecedented biological reduction step.

The exclusive retention of the H_B-ligand in the starter units of terpenes derived from **2a** and **3a** is a characteristic branching signature of the non-mevalonate pathway, which can be detected in its unperturbed form only in organisms in which the IPP-DMAPP isomerase is either absent or of low and dispensable activity [as is the case for *E. coli* (139)]. It is important to note that the original picture gets more and more blurred by successive increases in the competitiveness of the isomerase and the prenyltransferase(s) for their common substrates. Since the two enzymes engage the same H-ligand of IPP (H_B in **2a**) in their reactions [135, 140], isomerization of preformed **2a** will result in the loss of H_B and formation of the **3b** form of DMAPP (a fact which will be perceived as an apparent loss of H_B from **3a**), while isomerization of the original **3a** will lead to **2b**, which is expected to loose its H_W and retain H_B in the subsequent elongation step.

In the extreme case of a complete equilibration preceding the elongation process, any label associated with H_B will be lost during elaboration of the polyprenol intermediates, while at the same time the total label from the original pool of **3a** will be spread evenly over all the C_5 units of the final compound; a similar even spread of the label is expected in experiments with substrate bearing a label in the H_Y position. The first situation has been observed for the biosynthesis of phytoene and plastoquinone in BY-2 tobacco cells [141], the second is exemplified by the formation of hopanoid triterpenes in *Z. mobilis* [136]. With systems in which the isomerase and the prenyltransferase(s) are competing for their substrates with comparable efficiencies, feeding of precursors with H_B = D will result in preferential but not exclusive retention of the label in the starter derived subunit of the final products (for a possible case cf. [142]). Analysis of the associated apparent loss of deuterium caused in the starter unit by the partial equilibration with IPP is greatly facilitated by the use of doubly labelled substrates in which a ^{13}C-reporter nucleus is exploited for the accurate determination of the deuterium labelling pattern [134, 142].

The discovery of the unique branching signature of the non-mevalonate route to IPP and DMAPP has eventually paved the way for a realistic interpretation [142, 143] of the strikingly consistent but hitherto unexplained anomalies detected in a critical analysis of the natural abundance ^2H-NMR spectra of monoterpene hydrocarbons [144, 145].

Synthesis of non-mevalonate pathway intermediates

For the investigations of the basic non-mevalonate pathway as well as for mechanistic aspects of downstream terpenoid pathways, it is important to provide convenient synthetic routes affording the pathway intermediates in unlabelled and, even more important, in isotope-labelled form.

Feeding of isotope-labelled 1-deoxy-D-xyxlulose played an important role in the identification of the non-mevalonate pathway in certain bacteria and plants [15, 18, 25, 146]. Now, it is clear that the 5-phosphate of 1-deoxy-D-xyxlulose enters the pathway and that the incorporation of the unphosphorylated carbohydrate into terpenoids depends on the presence of an appropriate kinase in the experimental systems under study. In *E. coli*, D-xylulokinase specified by the *xylB* gene has been shown to convert 1-deoxy-D-xylulose into its 5-phosphate [101, 119]. Similar enzymes are obviously present in certain plant cells which can utilize exogenous 1-deoxy-D-xylulose.

Several methods for the chemical synthesis of 1-deoxy-D-xylulose have been described [24, 138, 142, 147–155]. In principle, they can be used for the preparation of isotope-labelled samples, and the incorporation of deuterium is achieved relatively easily. However, the preparation of ^{13}C-labelled material by these methods would be rather laborious.

1-Deoxy-D-xylulose has also been obtained from D-glyceraldehyde and pyruvate using crude cell extracts of *E. coli* [156]. The method can be used for the preparation of ^{13}C-labelled 1-deoxy-D-xylulose with commercially available ^{13}C-pyruvate as substrate. The synthetic carbohydrate can be converted to 1-deoxy-D-xylulose 5-phosphate using recombinant D-xylulokinase as catalyst (see above).

More recent synthetic strategies use recombinant 1-deoxy-D-xylulose 5-phosphate synthase as catalyst for the preparation of 1-deoxy-D-xylulose 5-phosphate from glyceraldehyde 3-phosphate and pyruvate as substrates [57]. Glyceraldehyde 3-phosphate can be prepared in situ from a wide variety of commercially available glucose isotopomers using commercially available glycolytic enzymes as catalysts. The reactions can be carried out as one-pot reactions with a minimum of experimental effort. Combinatorial strategies have been worked out which permit the synthesis of virtually every conceivable isotopomer of 1-deoxyxylulose 5-phosphate carrying ^2H, ^3H, ^{13}C or ^{14}C [57].

2*C*-Methyl-D-erythritol 4-phosphate has been prepared synthetically via 13 reaction steps with an overall yield of 7% [157]. The method was designed to introduce tritium at C-1; the preparation of other isotopomers would not be easily achieved with this approach. By comparison with this and other synthetic methods [158–160], enzyme-assisted combinatorial synthesis is again the method of choice for the preparation of a wide variety of isotopologs from glucose and pyruvate as typical starting materials [161]. Again, the multistep reactions can be carried out as one-pot reactions with very high overall yield.

Similarly, the later pathway intermediates **15** and **17** (cf. fig. 7) can be obtained with or without isotope label by appropriate enzyme-assisted synthesis strategies [162, 163]. The one-pot reaction leading to **17** involves 10 reaction steps and 15 enzymes. The reaction proceeds with near quantitative yield and with perfect regio- and stereocontrol [163]. Preparing the labelled target compound by conventional synthetic procedures [164] would be both highly laborious and costly.

The use of metabolic pathway enzymes for the efficient preparation of natural products via multistep one-pot reactions has been described in some detail because the general approach could represent a model for a wide variety of problems in natural products chemistry.

Seven different synthesis methods for the preparation of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**) have been reported within the short period since its discovery [165–171]. Some of these methods (169–171) allow the synthesis of isotope-labelled **18**.

Distribution of isoprenoid pathways in microorganisms

The completely sequenced genomes of about 150 microbial species in conjunction with biochemical studies afford a relatively detailed picture of the occurrence of the two isoprenoid pathways (cf. table 2). All known archaeal genomes contain genes with similarity to three mevalonate pathway genes, i.e. *hmgs* specifying 3-hydroxy-3-methylglutaryl-CoA synthase, *hmgr* specifying 3-hydroxy-3-methylglutaryl-CoA reductase and *mk* specifying mevalonate kinase, whereas genes coding for phosphomevalonate kinase (Pmk protein), diphosphomevalonate decarboxylase (Dpmd protein) and isopentenyl diphosphate isomerase (class I Idi protein) from eukaryotes are not found (table 2, fig. 1). Since it is well established by in vivo studies that archaea synthesise terpenoids via the mevalonate pathway [172–174], it must be assumed that these three enzymes have evolved independently in different taxonomic kingdoms. Indeed, one of the missing enzymes, an unusual isopentenyl diphosphate isomerase (class II) was recently identified [175].

Most completely sequenced eubacterial genomes exclusively comprise genes for the non-mevalonate pathway. However, the spirochaete *Borrelia burgdorferi* and Gram-positive cocci, e.g. *Staphylococcus aureus*, carry complete sets of mevalonate pathway genes, including the gene for a class II isopentenyl diphosphate isomerase. As an exception, the genome of the Gram-positive *Listeria monozytogenes* contains complete sets of mevalonate and non-mevalonate pathway genes together with a class II isopentenyl diphosphate isomerase.

Streptomyces coelicolor and *Streptomyces avermitilis* have complete sets of non-mevalonate pathway genes. In line with the genetic data, various terpenoids from actinomycetes have been shown by incorporation studies to originate via the non-mevalonate route. On the other hand, some *Streptomyces sp.* possess enzymes of the mevalonate pathway. This explains earlier in vivo experiments showing that both the non-mevalonate as well as the mevalonate pathway contributed to the biosynthesis of terpenoids in certain actinomycetes [54; reviewed in 176].

No genes with similarity to those of either the mevalonate pathway or the alternative pathway were found in *Mycoplasma genitalium, Mycoplasma pneumoniae, Rickettsia provazekii* and *Ureaplasma urealyticum*. As an exception, the genome of *Mycoplasma penetrans* carries genes for the non-mevalonate pathway. *R. provazekii* and *U. urealyticum* are obligatory intracellular parasites. *M. genitalium* has a highly specialized extracellular lifestyle on mucosal surfaces; the genome comprises only 484 genes believed to code for proteins. It is as yet unknown whether these highly specialized microorganisms obtain terpenoids from their mammalian hosts or do not require terpenoids at all. Notably, however, *Buchnera aphidicola* comprises a complete set of non-mevalonate pathway genes in its genome with 564 protein-coding genes.

Several minor anomalies in the distribution of orthologs should be noted. *Vibrio cholerae* has a full set of genes for the non-mevalonate pathway and a single gene with similarity to the *hmgr* gene of the mevalonate pathway. The hyperthermophilic archaeon *Pyrococcus hirokoshii* shows the mevalonate pathway enzymes characteristic of archaea and carries a putative *ispD* ortholog of the non-mevalonate pathway. The functional status of these genes with an anomalous distribution is unknown. They may be the result of lateral gene transfer. Some eubacteria with full sets of non-mevalonate pathway genes, e.g. *E. coli*, *Synechocystis* sp., *B. subtilis, Deinococcus radiodurans* and *M. tuberculosis*, possess orthologs of the genes specifying either isopentenyl diphosphate isomerase class I or II (*idiI* or *idiII*, table 2). Since IPP and DMAPP can be synthesized independently by the catalytic action of the IspH protein, it can be assumed that the isomerases serve as nonessential salvage pathway proteins in these organisms; indeed, *idiI* deletion mutants of *E. coli* were found to be viable on minimal medium [139].

Non-mevalonate pathway enzymes as antibacterial targets

The rapidly progressing resistance development in all microbial pathogens against all currently available antibiotics is one of the most pressing human health problems. The situation is aggravated by the increasingly frequent occurrence of multiresistent organisms. Whereas the mechanism of antibiotics resistance has been investigated in considerable detail, little has been achieved with regard to the implementation of preventive measures. Even the agricultural use of antibiotics in subtherapeutic doses in order to promote the weight gain of livestock continues in most countries despite its documented risks for human health [177]. The exploration of novel antibiotics targets and the development of novel antibiotics is therefore a matter of maximum urgency.

Only a relatively small number of bacterial gene products are targeted by antibiotics in current use which interact predominantly with the biosynthesis of macromolecular components of the bacterial cell (DNA, RNA, proteins, cell wall). Metabolic enzymes have played a minor role as antibiotic targets with the exception of folic acid biosynthesis enzymes, which are essential because folate coenzymes cannot be efficiently acquired by pathogenic bacteria due to the absence of an appropriate uptake system. Similarly, the terpenoid biosynthesis genes are essential because many bacterial pathogens are apparently unable to acquire terpenoids from their environment.

Whole genome sequencing of numerous pathogenic bacteria has enabled the systematic search for essential genes by gene targeting. Approximately 30 *E. coli* genes [178] with widely distributed orthologs have been identified as essential, among them all the non-mevalonate terpenoid genes described above. Many of these potential target genes have as yet no known function. On the other hand, the deoxyxylulose pathway enzymes have already been studied in considerable detail. This information could serve as a basis for high-throughput screening of chemical libraries as well as for rational drug development.

The non-mevalonate pathway is exclusively used for terpene biosynthesis by the majority of pathogenic bacteria, including *Mycobacterium tuberculosis* and *Helicobacter pylori*. As exceptions, certain Gram-positive cocci, such as *Streptococcus* and *Staphylococcus*, make their terpenes via mevalonate [105, 179, 180], *Listeria monozytogenes* has apparently complete sets of enzymes for the mevalonate pathway as well as for the non-mevalonate pathway, and *Rickettsia* has no enzymes for the synthesis of IPP/DMAPP. In *E. coli*, the deoxyxylulose phosphate pathway genes were shown to be essential; bacteria carrying deletions of any of these genes can be rescued by exogenous mevalonate after implementation of mevalonate pathway genes [121, 126, 181].

In summary, the deoxyxylulose phosphate pathway enzymes appear as highly attractive targets for the design of novel antiinfective drugs. It is also advantageous that the deoxyxylulose phosphate pathway enzymes are not present in mammalian cells, and aspects of selectivity need not be considered in drug design.

Distribution of isoprenoid pathway genes in eukaryotic species

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the microsporidian intracellular parasite *Encephalitozoon cuniculi*, the cryptomonad *Guillardia theta* and all completely sequenced animal species (*Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Drosophila melanogaster, Anopheles gambiae*, *Caenorhabditis elegans)* have complete sets of mevalonate genes, and genes of the alternative pathway are absent (National Center for Biotechnology Information) (table 2). This complements the ample biochemical evidence documenting that animals exclusively use the mevalonate pathway.

In some protozoal genomes, e.g. *Leishmania major* and *Trypanosoma cruzei*, orthologs of mevalonate pathway genes were found and characterized [182, 183]. On the other hand, putative orthologues of the non-mevalonate pathway genes were also reported to be present in the genome of *L. major* [184]. Clearly, further molecular and functional studies on isoprenoid biosynthesis in parasites, such as *Leishmania* and *Trypanosoma*, are necessary.

The apicomplexan parasite *Plasmodium falciparum*, the causative agent of malaria, exclusively has orthologs of the deoxyxylulose phosphate pathway genes [185]. Incompletely sequenced genomes of other apicomplexan protozoa (e.g. *Plasmodium vivax*, *Crytosporidium parvum* and *Toxoplasma gondii*) also show orthologs of the non-mevalonate pathway, but not of the mevalonate pathway. All reported isoprenoid biosynthesis genes from *Plasmodium* specify long N-terminal leader sequences [186], which are believed to target the enzymes to the apicoplast, an unusual cellular organelle with similarity to plant plastids (for review see [187]). It has therefore been proposed that the malaria parasites are more closely related to plants than to animals.

Plants use the mevalonate pathway in the cytoplasm for the biosynthesis of sterols and the non-mevalonate pathway in chloroplasts for the biosynthesis of a wide variety of mono- and diterpenes and higher isoprenoids comprising essential metabolites such as carotenoids and the phytol side chain of chlorophyll (see also next section). The *A. thaliana* genome shows a complete set of genes for both the mevalonate as well as the non-mevalonate pathway (table 2).

Genes from the non-mevalonate pathway have been sequenced in a variety of other plant species (table 1). In line with the intracellular topology of the two pathways, the open reading frames of all non-mevalonate pathway enzymes carry N-terminal sequences with a length of 60–100 amino acid residues which fulfil the criteria for chloroplast-targeting sequences. On the other hand, the mevalonate pathway genes of plants do not comprise targeting sequences, in line with their cytoplasmic location.
Not surprisingly, the full-length open reading frames for the non-mevalonate pathway genes of plants and *P. falciparum* could not be expressed in functional form in recombinant bacteria, whereas 'pseudomature' proteins with appropriate N-terminal truncation of the putative chloroplast or apicoplast-targeting sequence can be expressed to high level in functional form in recombinant *E. coli* strains [99, 108, 112].
It is generally accepted that chloroplasts originated by engulfment of blue-green algae, resulting in endosymbiosis. The genetic and biochemical aspects of the two terpenoid pathways are well in line with this hypothesis. The plant cytoplasm, which is equivalent to the intracellular compartment of the archaeal progenitor, has remained the site of the mevalonate pathway. The chloroplasts continue to use the non-mevalonate pathway enzymes supposed to have been present in the ancestral blue-green algae, although the cognate genes have been relocated to the nuclear genome, in parallel with the vast majority of the primordial endosymbiont genes.

Non-mevalonate pathway enzymes as antimalarial targets

Malaria has an estimated death toll of 1–3 million per year [188]. It is also a dominant source of morbidity, with an estimated number of 300–500 million infections per year. As in bacterial pathogens, the development and spreading of resistance is progressing rapidly.
No mevalonate pathway orthologs are present in the *P. falciparum* genome [185]. Thus it appears certain that the parasite exclusively uses the deoxyxylulose pathway for terpenoid biosynthesis.
The *ispC* and *ispF* genes could be expressed in recombinant bacterial host strains [89, 112]. The 2*C*-methyl-D-erythritol 4-phosphate synthase (IspC protein) of *Plasmodium falciparum* is inhibited by fosmido-mycin (**22**) and its homolog **23** (Fig. 9B) [89]. Mice in-fected with *Plasmodium vinckii*, a relative of the ma-laria parasite, could be cured by **22** [89]. These data firmly establish the deoxyxylulose pathway as a promising antimalarial target, and indeed, clinical trials using fosmidomycin in combination with clindamycin were successful in the treatment of malaria [189].

Non-mevalonate pathway enzymes as herbicide targets

Homozygous mutants of *A. thaliana* lacking 1-deoxyxylulose 5-phosphate synthase were not able to synthesise carotenoids and chlorophylls in sufficient amounts for normal growth [65]. Obviously, mevalonate-derived cytosolic terpenoid precursors could not contribute sufficiently by crosstalk to the formation of carotenoids and the phytol side chain of chlorophyll in the plastids of *Arabidopsis*. Indeed, in vivo experiments showed that the non-mevalonate pathway is the predominant source of the phytol side chain of chlorophyll and of carotenoids [25, 43, 146]. Hence, the pathway provides essential components of the light-harvesting apparatus and for photoprotection, and all enzymes of the non-mevalonate pathway are potential targets for novel herbicides. Not surprisingly, plants are therefore susceptible to the IspC inhibitor, fosmidomycin (**22**) [90, 190, 191].

Immunomodulation by non-mevalonate pathway intermediates

It has been known for some time that 'phosphoantigens' stimulate the proliferation of $\gamma\delta$ T lymphocytes expressing Vγ9/Vδ2 receptors [192, 193] in a process called innate immune recognition, which plays an important role in the early detection of pathogens [194]. Among the group of phosphoantigens, IPP and DMAPP were relatively potent stimulators [195]. More recently, it was shown that 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**18**) stimulates $\gamma\delta$ T cell proliferation at much lower concentrations, in the picomolar range [165, 196, 197].
The data suggest that the non-mevalonate pathway intermediate **18** serves as pathogen-associated molecular pattern which signals the presence of bacteria or protozoa to the mammalian immune system. This finding could provide the basis for the use of the natural product **18** as an immune-modulating drug [196].

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